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Cdc25 Regulates the Phosphorylation and Activity of the *Xenopus* cdk2 Protein Kinase Complex*

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The *Xenopus* cdk2 gene encodes a 32-kDa protein kinase with sequence similarity to the 34-kDa product of the cdc2 gene. Previous studies have shown that the kinase activity of the protein product of the cdk2 gene oscillates in the *Xenopus* embryonic cell cycle with a high in M-phase and a low in interphase. In the present study cdk2 was found not to be associated with any newly synthesized proteins during the cell cycle, but the enzyme did undergo periodic changes in phosphorylation. Upon exit from metaphase, cdk2 became increasingly phosphorylated on both tyrosine and serine residues, and labeling on these residues increased progressively until entry into mitosis, when tyrosine residues were markedly dephosphorylated. Phosphopeptide mapping of cdk2 demonstrated the major sites of phosphorylation were in a phosphopeptide with a pI of 3.7 that contained both phosphoserine and phosphotyrosine. This phosphopeptide accumulated in egg extracts blocked in S-phase with aphidicolin and was not evident in cdc2 immunoprecipitated under the same conditions. Under the same conditions cdc2 was phosphorylated primarily on a phosphopeptide containing both phosphothreonine and phosphotyrosine residues, most likely threonine 14 and tyrosine 15. Affinity-purified human GST-cdc25 was able to dephosphorylate and activate cdk2 isolated from interphase cells. Phosphopeptide mapping demonstrated that the phosphate was specifically removed from the same phosphopeptide identified as the major *in vivo* site of phosphorylation. These results demonstrate that cdk2 is regulated in the cell cycle by phosphorylation and dephosphorylation on both serine and tyrosine residues. Moreover, the increased phosphorylation of cdk2 in aphidicolin-blocked extracts and the ability of cdc25 to mediate cdk2 dephosphorylation *in vitro* suggest the possibility that cdk2 is part of the mechanism ensuring mitosis is not initiated until completion of DNA replication. It also implies cdc25 may have other functions in addition to the regulation of cdc2 kinase activity.

A major advance in cell cycle research in the past several years has come from the elucidation of the function of the *cdc2/CDC28* gene product. This advance stemmed from the discovery that cdc2 is the catalytic subunit of an enzyme known as maturation-promoting factor (Lohka *et al.*, 1988; Gautier *et al.*, 1988; Dunphy *et al.*, 1988). This enzyme had been identified in meiosis and mitosis from a wide range of cell types as sufficient to catalyze the entry of G_2-arrested frog oocytes into meiosis in the absence of new protein synthesis (Kishimoto *et al.*, 1982). A kinase related to the cdc2 gene product was also subsequently identified as a component of the mammalian growth-associated histone H1 kinase, whose activity correlates with chromosome condensation and the proliferative state of cells (Langan *et al.*, 1989). Since the cdc2 gene controls entry into mitosis in cells from yeast to humans, its regulation is clearly highly conserved and of fundamental importance in biology.

Active cdc2 kinase is a complex of the cdc2 gene product with a cyclin (Lohka *et al.*, 1988; Draetta *et al.*, 1989; Labbé *et al.*, 1989; Gautier *et al.*, 1990). The cyclins are characterized by their periodic accumulation and destruction (Minshull *et al.*, 1989), and their association with $p34^{cdc2}$ is necessary for the activation of cdc2 kinase and for entry of cells into mitosis (Murray and Kirschner, 1989). Cyclins fall into two general classes, G_2/M cyclins (A and B) and G_1 cyclins (C, D, and E) (Hunter and Pines, 1991; Pines and Hunter, 1991a, for review). Purified maturation-promoting factor is a complex of $p34^{cdc2}$ and cyclin B (Gautier *et al.*, 1990; Hunter and Pines, 1991; Labbé *et al.*, 1989; Meijer *et al.*, 1989). Cyclin A also forms active kinase complexes with $p34^{cdc2}$, although the kinetics of cyclin A/cdc2 kinase activation and inactivation differ from the cyclin B complexes (Minshull *et al.*, 1990; Walker and Maller, 1991). In addition to a requirement for cyclin association, activation of cdc2 kinase involves a complex series of phosphorylation and dephosphorylation events on the cdc2 subunit. In the case of cyclin B, newly synthesized protein associates with $p34^{cdc2}$ and induces its phosphorylation on Thr^{14} and Tyr^{15} in the ATP-binding site to form a catalytically inactive complex (Solomon *et al.*, 1990; Parker *et al.*, 1991). This complex is then abruptly activated at the G_2/M boundary by an obligatory dephosphorylation of Thr^{14} and Tyr^{15}. In *Schizosaccharomyces pombe* only Tyr^{15} is phosphorylated, and substitution of this residue with phenylalanine results in premature entry into mitosis (Gould and Nurse, 1989), indicating this dephosphorylation event is a key step in entry into mitosis. The timing of Tyr^{15} dephosphoryl-

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ation and cdc2 kinase activation is dependent upon the completion of DNA replication, as the presence of unreplicated DNA blocks tyrosine dephosphorylation and leads to accumulation of the inactive tyrosine-phosphorylated complex (Dasso and Newport, 1990; Kumagai and Dunphy, 1991).

From genetic studies, the *wee1*$^+$ gene is known to encode a dose-dependent inhibitor, and the *cdc25*$^+$ gene a dose-dependent activator, of the cdc2 kinase (Russell and Nurse, 1986, 1987). Recent studies show that direct phosphorylation/dephosphorylation of Tyr^{15} in p34^{cdc2} underlies these dose-dependent effects. The cyclin/cdc2 complex is a substrate for the dual specificity kinase wee1, which phosphorylates Tyr^{15} in cdc2 (Parker *et al.*, 1991, 1992), and the inactive complex can be activated *in vivo* and *in vitro* by the action of the cdc25 gene product, which has been shown to possess intrinsic tyrosine phosphatase activity (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Lee *et al.*, 1992; Strausfeld *et al.*, 1991).

A protein with a high degree of sequence identity to p34^{cdc2}, originally known as Eg1 but now termed cyclin-dependent kinase 2 or cdk2 (Paris *et al.*, 1991), has been studied recently in *Xenopus* eggs, where it is present both as a high molecular weight complex with histone H1 kinase activity and as a catalytically inactive monomer (Gabrielli *et al.*, 1992). In yeast it is well established that *cdc2*$^+$ regulates the cell cycle at both the G_1/S and the G_2/M restriction points. *Xenopus* cdk2 will not complement mutations in *cdc2*$^+$ in *S. pombe* or CDC28 in *S. cerevisiae* that involve G_2/M regulation (Paris *et al.*, 1991). However, when co-expressed with a human G_1 cyclin E, *Xenopus* cdk2 will substitute for CDC28 in *S. cerevisiae* at the G_1/S restriction point (Koff *et al.*, 1991), suggesting that in higher eukaryotes at least some aspects of G_1/S regulation involve cdk2 rather than cdc2.

In embryonic cell cycles, the activity of cdk2 oscillates with a periodicity similar to that of cdc2, but with a more modest change in activity (only 2–3-fold elevation in M-phase compared with >10-fold for cdc2) (Gabrielli *et al.*, 1992). In mammalian cells some fraction of cyclin A has been reported to be associated with cdk2 (Tsai *et al.*, 1991, Elledge *et al.*, 1992; Rosenblatt *et al.*, 1992). However, in *Xenopus* eggs cdk2 is apparently not associated with either of the mitotic cyclins, and the kinetics of its activation and inactivation are not as abrupt as observed for cyclin B/cdc2 complexes (Gabrielli *et al.*, 1992; Solomon *et al.*, 1990; Minshull *et al.*, 1990).

In this paper, we have investigated the mode of regulation of the *Xenopus* cdk2 H1 kinase. Our results indicate that the protein kinase activity of cdk2 is regulated by phosphorylation of the kinase subunit itself. A single major phosphopeptide from cdk2 labeled in egg extracts has been identified, and we demonstrate that *in vitro* the cdc25 phosphatase catalyzes dephosphorylation of this phosphopeptide coincident with activation of the kinase to levels similar to those seen *in vivo* at mitosis.

MATERIALS AND METHODS

Preparation of Eggs and Extracts—Cytostatic factor/metaphase-arrested extracts were prepared by a modification of the method of Lohka and Maller (1985) as described in Murray *et al.* (1989) except that leupeptin was omitted from all buffers. Aliquots were taken at the indicated times, and sequential cdk2 and p13-Sepharose precipitations for H1 kinase assays were performed as described previously (Gabrielli *et al.*, 1992), using a buffer containing 10 mM β-glycerophosphate, 5 mM NaF, 0.2% Triton X-100 and 0.1 M NaCl. In the case of ^{32}P-labeled extracts, the concentrations of added phosphocreatine and ATP were decreased to 0.38 mM and 50 μM respectively. $^{32}P_i$ (2 mCi/100-μl extract) was added at time zero, and in some experiments, a further 1 mCi of [γ-^{32}P]ATP was added 20 min prior to the termination of the reaction. Thin-layer chromatography of extracts demonstrated the added radioactivity equilibrated rapidly with ATP pools, and the specific activity remained constant for the duration of the labeling experiments. The samples were stored at −80 °C until analyzed.

Sperm pronuclei were prepared as described in Lohka and Maller (1985) and added to the extract to a final concentration of 2000 pronuclei/μl. Baculovirus-expressed GST-cdc25 was produced as follows: pML25 (Lee *et al.*, 1992) was partially digested with *Eco*NI and ligated to two partially overlapping oligonucleotides 5′-TCTAGATGCCTAT-3′ and 5-TATAGGCATCTAGA-3′ to generate pML25X. This step inserts a unique *Xba*I site and a new in-frame initiation site at the extreme N terminus of the GST gene. pML25-X was completely digested with *Xba*I and *Sma*I, and the 2.5-kb[1] insert containing the full length cdc25 gene fused to the GST gene was ligated into *Xba*/*Sma*I linear pVL1392 (Piwnica-Worms, 1990). The resulting plasmid pVLGST-25 was used to generate recombinant virus. Sf9 cells (3×10^6) were infected at an multiplicity of infection of approximately 10. At 40 h after infection, cells were harvested and frozen at −80. Cells were lysed by sonication in NETN buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/μl of each of leupeptin, pepstatin, chymostatin, and aprotinin). The lysates were rotated at 4 °C for 10 min, then centrifuged 10′ in a microcentrifuge and the supernatant (normally 500 μl) was added to 60 μl of glutathione-agarose beads. The supernatant and beads were mixed by rotation for 40′ min at 4 °C, then the beads were pelleted and washed with NETN buffer. The protein was used either bound to the glutathione-agarose beads or after elution with 10 mM glutathione.

Phosphoamino Acid Analysis and Tryptic Peptide Mapping of cdc2 and cdk2—After thawing, 100-μl samples of ^{32}P-labeled egg extract were diluted with 100 μl of extraction buffer (EB, Gabrielli *et al.*, 1992) containing 2 mM sodium vanadate and 50 mM *p*-nitrophenyl phosphate. The 200-ul samples were precleared by incubation with 50 μl of 50% protein A-Sepharose suspension at 4 °C for 30 min with gentle rotation, followed by centrifugation for 5 min at 15,000 × *g* in a microcentrifuge. The supernatant was incubated overnight with 15 μg of affinity-purified cdk2 antibody (Gabrielli *et al.*, 1992), precipitated with 40 μl of 50% protein A-Sepharose suspension for 1 h with gentle rotation, and collected by centrifugation in a microcentrifuge. The supernatant was then incubated with sheep anti-*Xenopus* cyclin B2 IgG fraction (20 μg; Izumi and Maller, 1991) for 4 h at 4 °C, followed by precipitation with 40 μl of a 50% suspension of protein G-Sepharose for 1 h at 4 °C. The immunoprecipitates were washed with RIPA buffer supplemented with 2 mM sodium vanadate, 0.2 mM ammonium molybdate, 80 mM β-glycerophosphate, 10 mM NaF, and 20 mM *p*-nitrophenyl phosphate as described in Gautier *et al.* (1989). The washed immunoprecipitates were electrophoresed on 12.5% SDS-polyacrylamide gels (Laemmli, 1970) and electrotransferred to either polyvinylidene difluoride or nitrocellulose membranes for phosphoamino acid analysis or tryptic phosphopeptide mapping, respectively. Transfer to polyvinylidene difluoride membranes was performed on an LKB semi-dry apparatus using 10 mM CAPS, pH 11, in 10% methanol as the transfer buffer (Matsudaira, 1987). The bands corresponding to labeled cdk2 and cdc2 were cut out, and two-dimensional phosphoamino acid analysis was performed as described by Kamps and Sefton (1989). Transfer to nitrocellulose was performed using a wet transfer apparatus (Hoeffer) in 192 mM glycine, 10 mM Tris, 0.01% SDS, and 20% methanol, overnight at 35 V with cooling. Quantitative transfer was obtained using these conditions. The ^{32}P-labeled bands were excised and blocked with 1% gelatin for 2 h at 37 °C. The nitrocellulose pieces were then washed with 50 mM ammonium bicarbonate, 5% CH_3CN and digested overnight with 50 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin in the same buffer at 37 °C. The membrane pieces were washed again with the same buffer, and the washes were pooled, dried, redissolved in 0.1 M acetic acid and dried again. The samples were dissolved in 20 μl of 0.1 M acetic acid and spotted onto small filter paper discs. The tryptic phosphopeptides were analyzed by isoelectric focussing in a Bio-Rad flat-bed apparatus using ultrathin-layer (0.2 mm) polyacrylamide gels containing a mixture of 1 part pH 3–10 and 4 parts pH 3–5 ampholines (Serva) with 5% (v:v) phosphoric acid as the anodic buffer and 1 M NaOH as the cathodic buffer. The sample discs were placed a third of the gel length from the cathode. The focussing was performed at 1500 V and 15 mA for 50 min, after which the gels were air-dried and autoradiographed. Resolved phosphopeptides were eluted from excised gel pieces overnight in 50 mM ammo-

[1] The abbreviations used are: kb, kilobase(s); SDS, sodium dodecyl sulfate; CAPS, 3-(cycloheylamino)propanesulfonic acid.

nium bicarbonate containing 5% CH_3CN, dried, hydrolyzed, and analyzed for phosphoamino acid content by two-dimensional thin-layer electrophoresis as described above. Thin-layer chromatography of bands eluted from isoelectric focussing gels was performed by spotting samples onto cellulose sheets (Kodak), and the chromatogram developed in *n*-butanol, pyridine, acetic acid, water (6:3.3:1:4).

In Vitro cdc25 Assays—Either unlabeled or ^{32}P-labeled cdk2 immunoprecipitates from DNA- and aphidicolin-blocked interphase extracts (Walker and Maller, 1991) were used as substrates for dephosphorylation by recombinant GST-cdc25 that had been purified by affinity chromatography on glutathione-agarose. Immunoprecipitates were incubated with the indicated amount of affinity-purified GST-cdc25 in 25 mM imidazole, pH 7.2, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin for 40 min at 25 °C. The H1 kinase activity of the unlabeled samples was assayed after washing the precipitates, and the sites in radiolabeled cdk2 dephosphorylated by cdc25 were determined by tryptic phosphopeptide mapping and autoradiography as described above.

RESULTS

It has been previously shown that cdk2 H1 kinase activity oscillates during the embryonic cell cycle (Gabrielli *et al.*, 1992). In this respect, it is reminiscent of cdc2 kinase, the activity of which has been shown to be regulated in part by the accumulation and destruction of its associated cyclin B subunit (Murray and Kirschner, 1989). Whereas cyclins A, B1, and B2 were easily detectable in cdc2 immunoprecipitates from [^{35}S]methionine-labeled egg extracts, no labeled proteins were specifically precipitated by cdk2 antibodies even with exposures 40 times longer than necessary to detect the cdc2-associated cyclins (Fig. 1). This is consistent with earlier results that demonstrated the activation of cdk2 H1 kinase at mitosis was not affected by inhibiting protein synthesis (Gabrielli *et al.*, 1992). We have also shown that active cdk2 exists as a high molecular weight complex. Gel filtration analysis of high speed supernatants from unfertilized eggs or ionophore-activated eggs at either interphase or the first mitotic metaphase showed significant amounts of cdk2 in an M_r 200,000 complex at all times (Gabrielli *et al.*, 1992 and data not shown). These results indicate that unlike cdc2, the activation and inactivation of cdk2 in the embryonic cell cycle are apparently not due to the synthesis and destruction of a cyclin-like subunit.

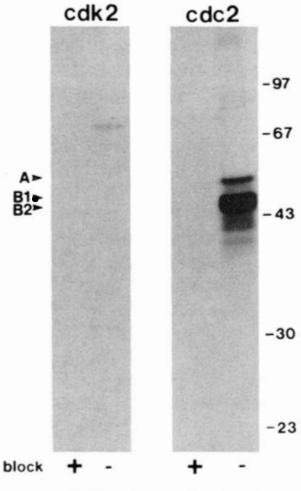


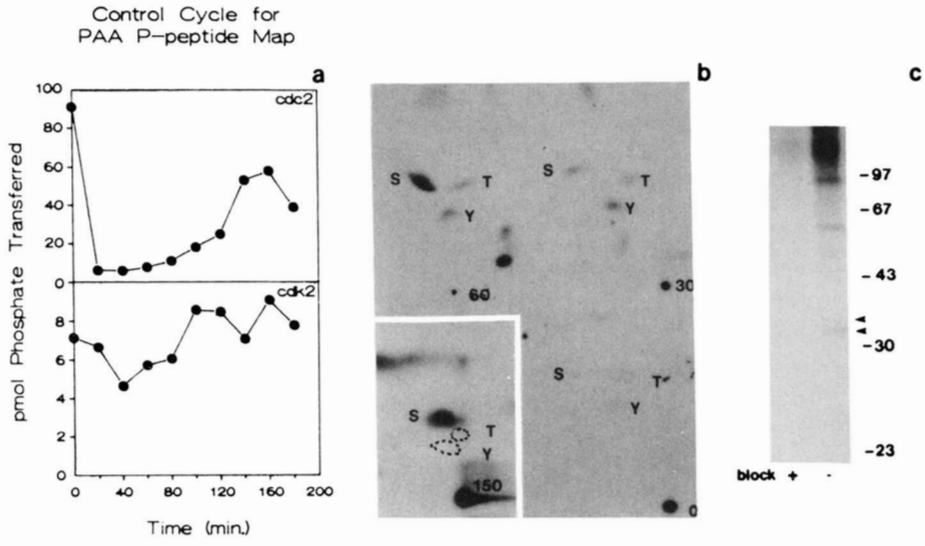
FIG. 1. **Immunoprecipitation of cdk2 and cdc2.** Cdk2 and cdc2 immunoprecipitates were prepared from [^{35}S]methionine-labeled egg extracts (0.5 mCi/ml) containing 2000 sperm pronuclei/μl 80 min after release from metaphase arrest by calcium addition. Immunoprecipitation was in RIPA buffer containing 0.4 M NaCl to extract all chromatin-bound complexes. The cdc2 antibody was raised against a synthetic peptide corresponding to the last 15 residues at the C terminus of *Xenopus* cdc2, and cdk2 antibody was raised against a peptide corresponding to the last 15 residues at its C terminus. Neither antibody cross-reacted with the other kinase. Each antibody was used at a dilution sufficient to completely immunodeplete its respective target protein from the extract. The ~80-kDa band in the cdk2 immunoprecipitate is probably nonspecific, as a band of similar molecular mass and intensity was also apparent in the cdc2 immunoprecipitate. Labeled bands were detected by fluorography for 2 weeks, and the faint 32-kDa band is cdk2. Blocking was performed by incubating the IgG with 2 μg of immunogen peptide per μg of antibody prior to immunoprecipitation. *Arrows* denote the position of cyclins A, B1, and B2 on the left, and molecular mass markers in kDa are on the right.

The Phosphorylation State of cdk2 Changes during the Cell Cycle—Given the precedent of direct regulation of cdc2 by phosphorylation, an obvious question concerned whether cdk2 was also regulated via phosphorylation. Extracts from unfertilized eggs that progressed through the cell cycle following addition of Ca^{2+} were incubated with 2 mCi of ^{32}P, and cdk2 was immunoprecipitated at various times (Fig. 2). It was routinely possible to obtain 500 cpm in cdk2/100 μl of extract by this method. Phosphoproteins of 95, 60, and a doublet of 32–33 kDa were specifically precipitated by the cdk2 antibody (Fig. 2*c*). The 32–33-kDa doublet could also be detected by PSTAIR immunoblotting of similar unlabeled cdk2 immunoprecipitates. The 95- and 60-kDa bands were precipitated by the cdk2 antibody even in samples from maturing oocytes where cdk2 was ablated by specific antisense oligodeoxynucleotide treatment (data not shown), suggesting that these two proteins were precipitated through direct interaction with the antibody and not indirectly through association with cdk2. The cyclin B2 antibody precipitated two major phosphoproteins, the 45-kDa cyclin B2 and the 34-kDa cdc2 (Izumi and Maller, 1991).

The phosphoamino acid content of cdk2 changed during the embryonic cell cycle (Fig. 2*b*). Phosphoserine was found throughout the cycle, but its relative intensity increased progressively after exit from metaphase (30 and 60 min, Fig. 2*b*). Phosphotyrosine was also observed in cdk2. This appeared in the earliest time point taken after exit from metaphase (30 min), paralleling the decrease in cdk2 activity. It was not detectable in first mitotic metaphase (150 min) when cdk2 activity had returned to maximal. Low levels of phosphothreonine were also observed and appeared to parallel the incorporation of phosphotyrosine. Thus, there are several phosphorylation sites, and it is likely that a number of different protein kinases and phosphatases may act on cdk2.

Tryptic Phosphopeptide Mapping of cdk2—The almost complete sequence identity of potential tryptic fragments of cdk2 and cdc2 that would contain known cdc2 phosphorylation sites, including Thr^{14}, Tyr^{15}, Thr^{161}, and Ser^{277} (Gould and Nurse, 1989; Krek and Nigg, 1991a), facilitated the analysis of phosphorylation sites in cdk2 by comparative tryptic phosphopeptide mapping. Both ^{32}P-labeled cdk2 and ^{32}P-labeled cyclin B2-associated cdc2 were isolated from metaphase-arrested extracts induced to cycle by calcium addition, using sequential immunoprecipitation with the appropriate antibodies. The cdk2 and cdc2 bands were excised from the gel, digested with trypsin, and analyzed by isoelectric focussing in ultrathin-layer polyacrylamide gels.

The major tryptic phosphopeptide derived from cdk2 at all stages of the cell cycle had a pI of 3.7 (Fig. 3*a*). This species was abundant during interphase (30 and 90 min) and then decreased with activation of cdk2 at metaphase (150 min). Densitometry of several experiments of this type revealed that the labeling of this band decreased 60–90% upon entry into mitosis. Phosphoamino acid analysis of this major cdk2 phosphopeptide revealed the presence of equal amounts of phosphoserine and phosphotyrosine (Fig. 3*c*, *panel 1*). Ex-



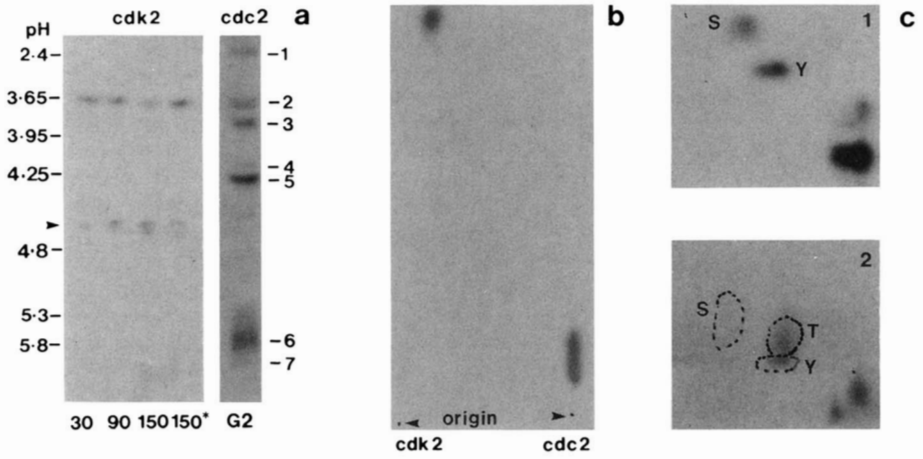


FIG. 2. **Changes in phosphoamino acid content of cdk2 during the cell cycle.** The H1 kinase activity of cdc2 and cdk2 was determined after Ca^{2+}-induced release of metaphase arrest in the extract as described under "Materials and Methods" (*a*). The phosphoamino acid content (*b*) of cdk2 immunoprecipitated from the extracts following release from metaphase arrest at the times indicated was determined by two-dimensional thin-layer electrophoresis and autoradiography as described under "Materials and Methods." *c*, Cdk2 was immunoprecipitated 150 min after calcium addition from ^{32}P-labeled extracts in the presence (*block* +) or absence (*block* −) of the immunogen peptide and analyzed by SDS-gel electrophoresis and autoradiography.

FIG. 3. **Phosphopeptide mapping of cdc2 and cdk2.** *a*, isoelectric focussing analysis of tryptic phosphopeptides generated from cdk2 and cyclin B2-associated cdc2 during the first mitotic cycle. ^{32}P-Labeled samples were taken at the indicated times, and cdk2 and cdc2 were isolated by immunoprecipitation and digested with trypsin as described under "Materials and Methods." As described in the text, bands corresponding to cdc2-derived phosphopeptides are numbered on the right from 1–7 from top to bottom, and the position of pI marker proteins are indicated on the left. The *arrow* indicates the position where samples were loaded on the gel. The radioactive bands evident at this position are precipitated during loading of the sample and are not seen consistently in other experiments (*cf.* Fig. 5*c*). *b*, thin-layer chromatography of the pI 3.7 cdk2- and cdc2-derived phosphopeptides (phosphopeptide 2 area). *c*, two-dimensional phosphoamino acid analysis of the major band of cdk2-derived phosphopeptide (*1*) and cdc2-derived band 1 (*2*).

tended autoradiography of the tryptic map revealed the presence of a number of minor tryptic phosphopeptides (not shown). These may account for the low level of phosphothreonine and the metaphase phosphoserine content of cdk2.

Isoelectric focussing of the cdc2-derived tryptic phosphopeptides showed a more complex pattern. One phosphopeptide (pI 3.7 cdc2 band 2) appeared to co-migrate with the major cdk2-derived phosphopeptide (Fig. 3*a*). However, when the two peptides were eluted and run in a second dimension on thin-layer chromatography, they were easily resolved (Fig. 3*b*) suggesting the phosphopeptides were not identical. The cdc2-derived band 2 may represent the Tyr^{15}-monophosphorylated tryptic fragment, based on its phosphoamino acid content and its insensitivity to dephosphorylation by protein phosphatase 1 (data not shown).

It was possible to tentatively identify the major site of mitotic regulation on cdc2, Thr^{14}/Tyr^{15}, as being contained in peptide 1, which had a pI of 2.4. Phosphoamino acid analysis of the phosphopeptide revealed only phosphothreonine and phosphotyrosine (Fig. 3*c*, *panel 2*), and it was detected in tryptic maps of cdc2 only during G_2 phase of the embryonic cell cycle (~90 min) just prior to the mitotic activation of cdc2 kinase (data not shown). Phosphopeptides in bands 3, 4, and 5 contained only phosphoserine, while some phosphotyrosine was also detected in band 7, and a low level of phosphothreonine in addition to phosphoserine was detected in band 6 (see Fig. 5*a*). The variability and more neutral pI of bands 6 and 7 suggest these may represent incomplete digestion products.

Unreplicated DNA has been demonstrated to inhibit the activation of cyclin B/cdc2, resulting in the accumulation of tyrosine-phosphorylated cdc2 in the cyclin B complex (Dasso and Newport, 1990; Kumagai and Dunphy, 1991; Walker and Maller, 1991). This appears to be due to the inhibition of Tyr^{15} dephosphorylation, and probably also Thr^{14} dephosphorylation by cdc25. Consistent with this notion, addition to 50 μg/ml of the DNA synthesis inhibitor aphidicolin in extracts containing pronuclei completely blocked the normal activation of cdc2 at metaphase, and, importantly, also blocked the activation of cdk2 (Fig. 4). Tryptic phosphopeptide maps of

cdk2 from such DNA/aphidicolin-blocked samples contained an elevated level of the pI 3.7 phosphopeptide (Fig. 3*a*, *150**). This was further evidence that phosphorylation of this tryptic fragment was closely associated with decreased cdk2 kinase activity. Only the phosphopeptide designated band 1 accumulated in cdc2-derived maps from similar samples (see Fig. 5*c*), consistent with the reported accumulation of phosphotyrosine on cdc2 under similar conditions (Kumagai and Dunphy, 1991) and the notion that the Thr^{14}/Tyr^{15} sites regulate the activation of cdc2 kinase at mitosis.

Regulation of cdk2 Phosphorylation and Activity by cdc25—Given the evidence described above that cdk2 was negatively regulated by tyrosine phosphorylation, it was important to evaluate whether cdc25 could act on the cdk2 complex. The addition of affinity-purified GST-cdc25 to DNA/aphidicolin-blocked interphase egg extracts resulted in the activation of cdc2 kinase as reported previously (Kumagai and Dunphy, 1991), but cdk2 was also activated to metaphase levels (Fig. 5*a*). There was no appreciable lag before activation of cdk2 kinase after addition of the cdc25, suggesting that cdc25 may act directly on cdk2 to dephosphorylate and activate the kinase. This direct mechanism was supported by the demonstration that cdk2 immunoprecipitates from DNA/aphidicolin-blocked extracts were activated for H1 phosphorylation by incubation with GST-cdc25 to levels 50% above control levels (Fig. 5*b*). Similar results were obtained with cyclin B2-immunoprecipitated cdc2 kinase from cdk2-immunodepleted samples, although the degree of activation varied from 50 to 200% depending on the experiment (Fig. 5*b*). The degree of activation of both cdk2 and cdc2 was less than that observed when cdc25 was added to the interphase-blocked extracts. This may be due in part to the lesser amount of cdc25 protein used in the *in vitro* activation (0.15 μg compared to 1 μg added to the extract) or to physical constraints in the immunoprecipitate.

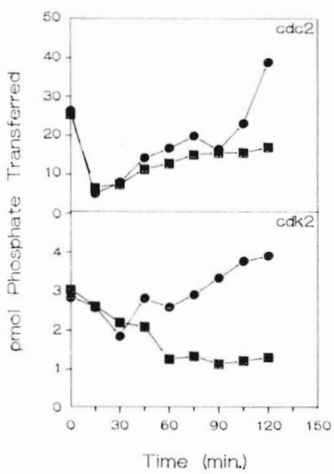


FIG. 4. **Inhibition of DNA synthesis blocks activation of both cdc2 and cdk2.** The cdk2 and cdc2 H1 kinase activities were assayed at various times after calcium addition to metaphase-arrested extracts containing 2000 pronuclei/μl in the absence (*circles*) or presence (*squares*) of 50 μg/ml aphidicolin.

Incubation of ^{32}P-labeled cdk2 and cyclin B2 immunoprecipitates from DNA- and aphidicolin-blocked extracts with GST-cdc25 resulted in the specific dephosphorylation of the cdk2 and cdc2 phosphoproteins (data not shown). Tryptic phosphopeptide mapping of the labeled cdk2 revealed the dephosphorylation of the pI 3.7 band, without the appearance of a new more basic phosphopeptide, suggesting that cdc25 dephosphorylated both serine and tyrosine in this peptide (Fig. 5*c*). The decrease in the pI 3.7 band after cdc25 treatment was quantified by densitometry in four separate experiments to be between 50 and 70%.

The putative Thr^{14}/Tyr^{15}-containing band 1 was the major site of cdc25 action on cdc2. The two most basic bands, 6 and 7, were also diminished, supporting the notion that they represent incomplete tryptic digestion products. Band 2 in cdc2 was also dephosphorylated. The other major cdc2 phosphopeptides were unaffected. The dephosphorylation of band 1 went to near completion and no new, more basic band representing a monophosphorylated peptide was detected, indicating the likelihood that cdc25 dephosphorylated both threonine and tyrosine residues in this peptide. The apparent ability of cdc25 to dephosphorylate all three types of phosphorylated residues is not unexpected, since cdc25 appears to be distantly related to a newly discovered class of "dual specificity" serine/threonine and tyrosine phosphatase (Gautier *et al.*, 1991 and references therein).

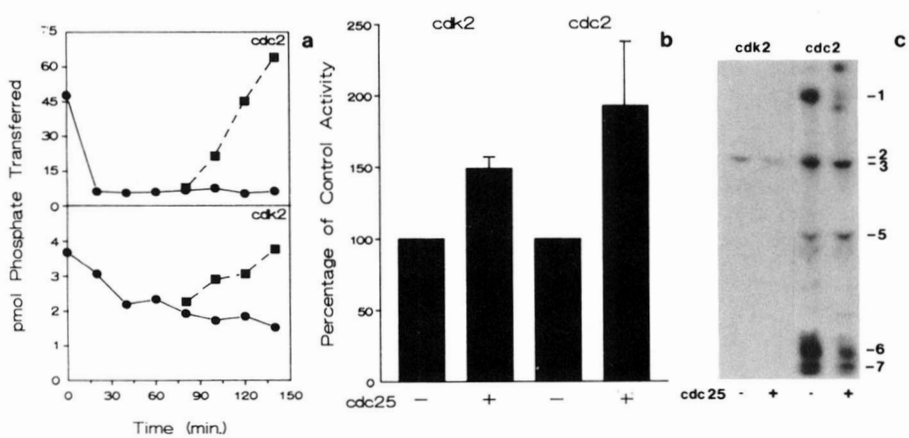


FIG. 5. **cdc25 dephosphorylates and activates both cdk2 and cdc2.** *a*, cdk2 and cdc2 H1 kinase activities were assayed from either DNA/aphidicolin-blocked interphase extracts (*circles*) or similar extracts to which purified GST-cdc25 (1 μg of protein; *squares*) was added after 80 min. *b*, immunoprecipitates of either cdk2 or cyclin B2-associated cdc2 from DNA/aphidicolin-blocked extracts (samples taken at 120 min) were incubated with either buffer alone or GST-cdc25 (0.15 μg of protein), then washed and assayed for H1 kinase activity. The values are the mean ± S.E. of five separate experiments. In some experiments the control addition was baculovirus-expressed GST protein purified by glutathione-Sepharose, using the procedure described for GST-cdc25. *c*, similar DNA/aphidicolin-blocked samples from ^{32}P-labeled extracts were immunoprecipitated as in *b* and incubated with either buffer or GST-cdc25 (1.2 μg) then analyzed by tryptic phosphopeptide mapping.

DISCUSSION

Similarities and differences in the regulation of cdk2 and cdc2 are evident from the data in this paper. In the *Xenopus* embryonic egg cell cycle, the synthesis and destruction of cyclin B has been shown to be a major controlling factor in the regulation of mitotic cdc2 kinase activity (Murray and Kirschner, 1989; Murray *et al.*, 1989), although the presence of unreplicated DNA will activate an otherwise dormant checkpoint (Dasso and Newport, 1990; Walker and Maller, 1991). Unlike cdc2 however, cdk2 in *Xenopus* eggs appears to form a stable complex with unidentified proteins,[2] and the activity of the complex is regulated by phosphorylation of the cdk2 protein and not by association with newly synthesized proteins (Fig. 1). This agrees with earlier results that demonstrated cdk2 activation was independent of protein synthesis (Gabrielli *et al.*, 1992). The failure to detect cdk2/cyclin A complexes in the *Xenopus* embryonic system is apparently at odds with recent results from a number of groups that have demonstrated that cdk2 is associated with cyclin A in somatic cell systems (Tsai *et al.*, 1991; Elledge *et al.*, 1992; Rosenblatt *et al.*, 1992). In fact, we have detected only cdc2 associated with cyclin A in *Xenopus* eggs. However, large differences in the association of cyclin A with cdc2 and cdk2 have been reported between adherent and suspension-grown HeLa cells and in various other cell types (Elledge *et al.*, 1992), suggesting that the relative proportion of cyclin A complexed with cdc2 and cdk2 varies with different cell types and with different growth conditions.

Our data indicate that the regulation of cdk2 kinase in the *Xenopus* embryonic cell cycle is due primarily to the specific phosphorylation and dephosphorylation of the cdk2 protein subunit. The major cdk2-derived tryptic phosphopeptide is a phosphoserine- and phosphotyrosine-containing species and was not evident in the cdc2-derived phosphopeptides. While we have only tentatively identified the cdc2-derived Thr^{14}/Tyr^{15}-containing phosphopeptide, it is clearly evident that this same site, which is conserved in the cdk2 sequence, is not the major site of cdk2 kinase regulation. Identification of the major cdk2 regulatory site will require more complete mapping and sequence analysis. We have also demonstrated that cdc25 acts directly on the unique phosphoserine- and phosphotyrosine-containing phosphopeptide to activate the cdk2 H1 kinase. The phosphorylation and subsequent dephosphorylation of this phosphopeptide most closely correlated with the inactivation and reactivation of the cdk2 complex during the cell cycle. These data together strongly suggest that the sites contained in this phosphopeptide are of major importance for cdk2 regulation.

Our experiments support the idea that the dephosphorylation of Thr^{14}/Tyr^{15} in cdc2 by cdc25 is a major regulatory mechanism for activation of the *Xenopus* enzyme. These data are in agreement with those of others who have shown that dephosphorylation of this site is temporally related to activation of the cyclin B-cdc2 complex at the G_2/M boundary of the cell cycle (Gould and Nurse, 1989; Solomon *et al.*, 1990; Krek and Nigg, 1991a) and that mutation of this site to nonphosphorylatable residues in fission yeast results in premature entry into mitosis (Gould and Nurse, 1989; Norbury *et al.*, 1991; Krek and Nigg, 1991b). As Tyr^{15} is the only site of tyrosine phosphorylation on cdc2 identified in this study and others (Gould and Nurse, 1989; Krek and Nigg, 1991a; Norbury *et al.*, 1991), our results also agree with other evidence that treatment of cdc2 with cdc25 results in the complete loss of phosphotyrosine from cdc2 (Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991; Lee *et al.*, 1992).

A significant difference between cdk2 and cdc2 regulation during the embryonic cell cycle is evident in the fact that cdk2 activity decreases only 2–3-fold in interphase, whereas cdc2 kinase decreases 10–20-fold. It was possible to reduce cdk2 activity to below the normal interphase level by addition of DNA/aphidicolin to extracts, but even under those conditions cdk2 kinase activity was not completely inhibited. These results could be accounted for by at least three possible explanations. The first is that two pools of cdk2 kinase exist, one that is highly regulated, and a second that is less sensitive or insensitive to regulation. A second possible explanation is that the interphase phosphorylation of cdk2 reduces the H1 kinase activity of the cdk2 complex, but does not completely inactivate it, in contrast to the case of cdc2 kinase (Parker *et al.*, 1992), where the cdc25-sensitive phosphorylation sites are directly in the ATP-binding site. Finally, only cdk2 in certain cellular compartments may be subject to inhibition. We are currently unable to differentiate between these possibilities.

The regulation of cdk2 raises some interesting questions about the activity of cdc25 during the cell cycle. In the frog embryonic system, the activation of cdk2 and cdc2 kinases occurs at a similar stage of the cell cycle although cdk2 is consistently activated slightly before cdc2. Several cdc25 homologs have been cloned by ourselves and others, although any differences in their kinetics of activation are not known at present. Recently, it has been reported that in somatic cells cdk2 is activated in early S-phase (Rosenblatt *et al.*, 1992), suggesting the possibility that at least some isoforms of cdc25 may be active very early in the cell cycle. This is unexpected from investigations of cyclin B-cdc2 regulation in which Tyr^{15}-phosphorylated cyclin B/cdc2 complexes accumulate until very near the G_2/M boundary (Gould and Nurse, 1989; Solomon *et al.*, 1990; Meijer *et al.*, 1991). An unknown event, possibly phosphorylation, then activates cdc25 to dephosphorylate and activate cdc2 kinase at the G_2/M boundary. Early activation of cdk2 could also reflect the relatively higher abundance of the cdk2 kinase complex early in the cell cycle compared to the level of cyclin B/cdc2. The timing of cyclin B/cdc2 dephosphorylation could also reflect changes in the localization of cdc25 or the movement of cyclin B/cdc2 into the nucleus at the onset of prophase as reported by Pines and Hunter (1991b) for cultured cells. Clearly more detailed investigation of the activity and regulation of various cdc25 isoforms during the cell cycle and localization of cdk2 is necessary to evaluate temporal differences in phosphorylation and dephosphorylation of cdk2 and cdc2.

We have shown that human cdc25 can act on frog cdk2. While the human enzyme modulates frog cdk2 activity in a manner similar to that observed *in vivo*, we cannot exclude the possibility that human cdc25 loses some essential specificity when used across species lines. An example of such a loss is the ability of human mitotic cyclins to functionally complement loss of budding yeast G_1 cyclins, whereas the yeast mitotic cyclin is unable to do so (Lew *et al.*, 1991). Thus it is possible that functional analogs of cdc25 exist in *Xenopus* eggs which specifically regulate cdk2 activity. A precedent for this is the discovery of the *wee1+* functional homolog, *mik1*, which can rescue a *wee*$^-$ mutant in *S. pombe* (Lundgren *et al.*, 1991). Data presented in that paper suggested that *mik1* may have a specific role in regulating the cdc2 prereplicative START function thought to be assumed by cdk2 in higher eukaryotes. Direct purification from *Xenopus* eggs of the phosphatase(s) that act on the cdk2 regulatory sites will be required to unequivocally establish which enzymes are acting *in vivo*.

[2] B. G. Gabrielli and J. L. Maller, manuscript in preparation.

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